

**REMARKS**

Figure 1 is a photograph showing the results of Northern blotting analysis showing a change in the amount of HSP12 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 2 is a photograph showing the results of Northern blotting analysis showing a change in the amount of DBP2 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 3 is a photograph showing the results of Northern blotting analysis showing a change in the amount of NSR1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 4 is a photograph showing the results of Northern blotting analysis showing a change in the amount of AAH1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 5 is a photograph showing the results of Northern blotting analysis showing a change in the amount of YKR075C mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 6 is a photograph showing the results of Northern blotting analysis showing a change in the amount of OLE1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 7 is a photograph showing the results of Northern blotting analysis showing a change in the amount of ACT1 mRNA obtained when the culture temperature is decreased from 30°C to 10°C. The culture

time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 9 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having a DBP2 promoter function is ligated to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 10 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having a DBP2 promoter function is ligated in the direction opposite to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 11 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HMT1 promoter function is ligated to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 12 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HMT1 promoter function is ligated in the direction opposite to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 13 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HSP12 promoter function is ligated to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 14 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA obtained when a DNA fragment having an HSP12 promoter function is ligated in the direction opposite to EGFP DNA and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 15 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA, which is obtained, when a DNA fragment having a modified DBP2 promoter function (right) obtained by removing a DNA sequence A (GCTCATCG) from a DNA fragment having a DBP2 promoter function comprising the above DNA sequence A and a native DNA fragment having a DBP2 promoter function (left) are used, and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 16 is a photograph showing the results of Northern blotting analysis showing a change in the amount of EGFP mRNA, which is obtained, when a DNA fragment having a modified HMT1 promoter function (right) obtained by removing a DNA sequence B (GAGATGAG) from a DNA fragment having an HMT1 promoter function comprising the above DNA sequence B and a native DNA fragment having a HMT1 promoter function (left) are used, and when the culture temperature is decreased from 30°C to 10°C. The culture time after a low temperature treatment is shown at the top. The amount of mRNA is represented by the density and size of dots in each lane.

Figure 17 is a photograph showing in the upper case, a plasmid construct comprising an ADH1 promoter, a TDH3 promoter, or a DNA fragment having an HSP12 cold-inducible promoter function, and in the middle and lower cases, the results of Northern blotting analysis, which is performed to compare the transcriptional activity of a DNA fragment having an HSP12 cold-inducible promoter function with the transcriptional activities of an ADH1 promoter and a TDH3 promoter in yeast. The amount of EGFP mRNA is represented by the density and size of dots in each lane.

Figure 18 is a photograph showing in the upper case, a plasmid construct comprising a TDH3 promoter or a DNA fragment having an HSP12 cold-inducible promoter function, and in the lower case, the results of

Western blotting analysis, which is performed to compare the protein-producing ability of a DNA fragment having an HSP12 cold-inducible promoter function with the protein-producing ability of a TDH3 promoter in yeast. The amount of an EGFP protein is represented by the density and size of dots in each lane.

Figure 19 is a photograph showing in the upper case, an expression plasmid construct, which is obtained by inserting an expression cassette comprising a DNA fragment having an HSP12 cold-inducible promoter function, the ORF of EGFP, and a CYC1 terminator, into pUG35 having a centromere as a replication origin, pYES2 having 2  $\mu$  as a replication origin, or pYEX-BX having 2  $\mu$  as a replication origin and having a weak leucine synthetase gene (*leu2-d*), from each of which an original promoter has been removed, and in the middle and lower cases, the results of Northern blotting analysis showing the fact that the ability of the transcriptional activation of a DNA fragment having an HSP12 cold-inducible promoter function does not depend on the structure of a plasmid in itself. The amount of EGFP mRNA is represented by the density and size of dots in each lane.

Figure 20 is a photograph showing the results of SDS-PAGE analysis showing the fact that the protein-producing ability of a DNA fragment having an HSP12 cold-inducible promoter function does not depend on the structure of a plasmid in itself. The amount of an EGFP protein is represented by the density and size of a band indicated with an arrow in each lane.

Figure 21 is a photograph showing the results of SDS-PAGE analysis showing the fact that the protein-producing ability of a DNA fragment having an HSP12 cold-inducible promoter function does not depend on the type of yeast strain *Saccharomyces cerevisiae*. The amount of an EGFP protein is represented by the density and size of a band indicated with an arrow in each lane.

Figure 22 is a photograph showing the results of SDS-PAGE analysis showing the fact that the protein-producing ability of an expression vector comprising a DNA fragment having an HSP12 cold-inducible promoter function is more excellent than that of the existing expression vector of yeast. The amount of an EGFP protein is represented by the density and size of a band indicated with an arrow in each lane.

Figure 23 is a photograph showing the results of SDS-PAGE analysis showing the fact that the protein-producing ability of an expression vector comprising a DNA fragment having an HSP12 cold-inducible promoter function is induced in a wide low temperature range. The amount of an EGFP protein is represented by the

density and size of a band indicated with an arrow in each lane.

Figure 24 is a photograph showing the results of Western blotting analysis showing the fact that a cassette comprising an HSP12 promoter, the ORF of EGFP, and a CYC1 terminator was incorporated into methylophilic yeast, *Pichia pastoris*, so that an EGRP protein was inducibly produced in *Pichia pastoris* at a low temperature.

Figure 25 is a photograph showing the results of Western blotting analysis showing the fact that an antifreeze protein RD3 is expressed as a soluble protein by a DNA fragment having an HSP12 cold-inducible promoter function. The amount of an RD3 protein is represented by the density and size of a band in each lane.

Figure 26 is a photograph showing results obtained by expressing two types of fluorescent proteins, ECFP and DsRed by cold induction using pLTex321.

Respectfully submitted,

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